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Nose-Only Versus Whole-Body Aerosol Exposure for Induction of Upper Respiratory Infections of Laboratory Mice*,†

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The effectiveness of two aerosol delivery systems, nose-only and whole-body, were compared using Swiss-Webster mice and two pathogens, *Klebsiella pneumoniae* and Venezuelan equine encephalitis (VEE) virus. With *K. pneumoniae* the median lethal dose (LD_{50}) and the mean time to death correlated with the inhaled dose. An LD_{50} value of 335 colony forming units (cfu) for nose-only exposure was significantly less than the LD_{50} value of 3741 cfu obtained for whole-body exposure. The LD_{50} values obtained with VEE virus for nose-only exposure [8 plaque forming units (pfu)] and whole-body exposure (11 pfu) were similar to each other. Following a 10-min nose-only exposure, concentrations of *K. pneumoniae* approximating 10^4/g were present after 24 hr in the upper respiratory tract (URT) and lungs. The numbers of bacteria reached a peak at 72 hr, when resolution of the infection began. Detectable levels of bacteria in the blood and tissues were delayed in mice given whole-body exposure, plus there was a decreased concentration of bacteria per gram of tissue. Major pathological lesions induced by *K. pneumoniae* were mild suppurative rhinitis and minimal suppurative bronchopneumonia. Viremia was greatest at 96 hr following aerosol exposure to VEE. Virus concentrations in the URT, lungs, cerebrum, spleen and mesenteric lymph nodes reached maximum titers earlier for mice exposed by nose-only than for mice exposed to whole-body aerosols. High virus concentrations were associated with necrosis of the lymphoid tissues which was the probable cause of death. Disease induced with VEE virus by both aerosol systems was identical.

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Introduction

Airborne transmitted diseases commonly are produced experimentally by intranasal instillation or by exposure to aerosol. Effective deposition of infectious microorganisms into the bronchioles and alveoli only can be attained via small particle aerosols. Thus, generation and delivery of infectious or toxic aerosols are fundamental to developing an understanding of the pathogenesis and immunogenesis of airborne transmitted diseases. The authors' studies of pathogenesis and immunogenesis have been conducted using aerosols in which the mass median diameter of the particles approximates 1.0-1.5 (μm) micrometers.

For a number of years, a dynamic aerosol system comprised of a Henderson tube modified by incorporation of an exposure box[1] has been used in the authors' laboratory for studies of airborne transmitted infectious or toxic agents. Results obtained with this system have been highly reproducible; however, the entire body of the animal is exposed to the aerosol. Consequently, routes of entry for the challenge agents other than the respiratory tract could not be excluded. Preening, as occurs with rodents, could result in infection being established through the gastrointestinal tract in addition to the respiratory tract and could yield pathogenesis differences.

The whole-body aerosol exposure system is not optimal for the accurate assessment of disease mechanisms or immune responses when the respiratory system is the purported primary portal of entry of the causative agent. The authors, therefore, initiated studies of delivery systems which would limit exposure principally, if not exclusively, to the respiratory tract. In the studies reported, the authors compared whole-body exposure with nose-only exposure using both a mouse-bacterium model and a mouse-virus model.

Materials and Methods

Mice

Outbred, female, Swiss-Webster mice (18-22 g), free of Sendai virus infection, were obtained from Harlan Laboratory Animals (Walkersville, Md.). Mice were maintained in appropriate cages and given water and ration pellets *ad libitum* throughout the study. Quality control evaluations showed the mice were essentially free of histologic lesions induced by any previous exposures to pathogens.

Microorganisms

The A-D strain (Type 1) of *Klebsiella pneumoniae* and the Trinidad strain of Venezuelan equine encephalitis (VEE) virus were used. Frozen seed stocks of *K. pneumoniae* were

*In conducting the research described, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

†The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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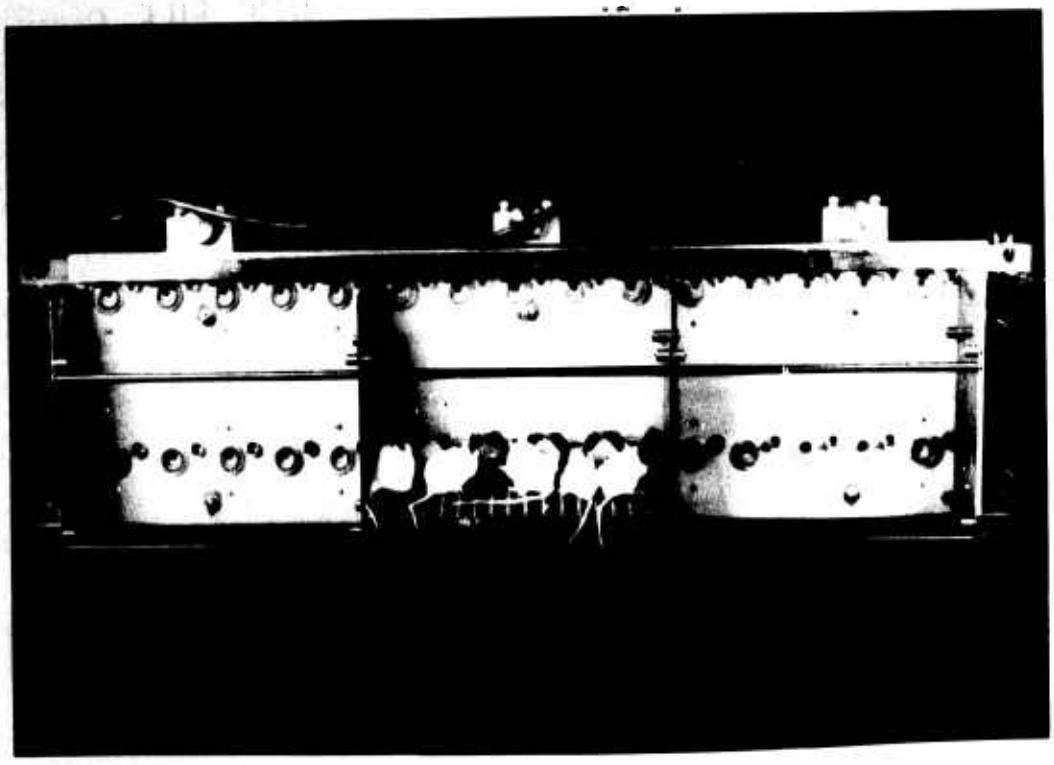

Figure 1 — Nose-only aerosol exposure system.

Figure 2 — Whole-body aerosol exposure system.

TABLE I
Comparison of Respiratory Dose Response to *Klebsiella pneumoniae* for Swiss-Webster Mice Exposed by Three Procedures

Challenge Type	MDTD[A] (> 1.0 LD_{50} Dose)	LD_{50}[B] (95% CL[C])
Nose-Only	3.1 - 5.0	335 (209-537)
Whole-Body	5.3 - 7.7	3741 (1473-9500)
Intranasal	2.3 - 4.2	6 (3-12)

[A]Mean days to death, geometric mean.
[B]Expressed as colony forming units (cfu)
[C]Confidence limit.

inoculated into flasks of trypticase soy broth and incubated at 37°C for 16 hr.[2,3] Desired working dilutions were made using cold trypticase soy broth. The stock virus preparation was a 10% (w/v) suckling mouse brain suspension in Eagle's minimal essential medium with Earles's salts and nonessential amino acids (EMEM), plus 10% fetal bovine serum (FBS) and antibiotics.

Respiratory Challenge

Nose-only exposures were accomplished using the inhalation system of Microbiological Associates (Bethesda, Md.) (Figure 1).[4,5] For whole-body studies, exposures were performed using a dynamic aerosol system[1] that was comprised of a Henderson-type aerosol transit tube modified by incorporation of a plastic animal-exposure box [26.7 cm wide × 52.1 cm high × 30.5 cm deep (10.5 in. wide × 20.5 in. high × 12.0 in. deep)] (Figure 2). Aerosol generation for both systems was achieved with a Collison disseminator (BGI, Inc., Waltham, Mass.).[6] Preliminary evaluations showed the mass median diameter of the particles generated was 1.2 μm to 1.6 μm, depending on the suspending medium used. The airflow was 4.0 Lpm through the nose-only system and 28.0 Lpm through the whole-body system. Aerosols were sampled for 5 min at the midpoint of the exposure period using standard all-glass impinger (AGI-30) samples.[7] Collection fluid in the samplers for *K. pneumoniae* consisted of 20.0 mL of trypticase soy broth containing 0.1% (v/v) antifoam (Dow-Corning Antifoam Y-30 Emulsion). For VEE virus the collection fluid was EMEM supplemented with 5% FBS, 100 units of penicillin/mL, 100 μg of streptomycin/mL, and 0.1% antifoam. Each animal was exposed for 10 min. Respiratory minute volumes were estimated using Guyton's formula.[8] Total inhaled doses were calculated from the minute volumes and the aerosol concentration of microorganisms delivered.

Microbial Assays

Quantitation of *K. pneumoniae* in suspension, aerosol samples and animal tissue specimens was by routine dilution and plating procedures.[2,3] Serial decimal dilutions were made in trypticase soy broth, then 0.1 mL of selected dilutions was spread onto the surface of each of three trypticase soy agar plates. After incubation at 37°C for 16 hr, the number of colonies was ascertained and concentrations were calculated in terms of colony forming units (cfu). Plaque assay procedures were used to quantitate VEE virus concentrations,[9] with minor modification. Confluent Vero cell monolayers in 6-well (9.6 cm^2/well) plastic plates were inoculated with 0.2 mL of inoculum, diluted in Hank's balanced salts solution containing 25 mmol HEPES (N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid) buffer, 2% FBS and antibiotics. Viruses were allowed to adsorb for 1 hr at 37°C, then the cultures were overlaid with 3.0 mL of medium containing Eagle's basal medium with Earle's salts, 4% FBS, 25 mmol HEPES buffer, antibiotics and 0.5% agarose. After the cultures were incubated at 37°C in a 5% CO_2 atmosphere for 48 hr, 3.0 mL of secondary medium — comprised of the same overlay medium plus neutral red at a final concentration of 1:10 000 — was added. Plaques were counted following an additional 24 hr incubation.

Infectivity Evaluation

Determinations were performed in triplicate. Groups of 10 mice were given each selected dose. In addition to exposure by the two aerosol systems, a series of mice in each test was challenged by intranasal instillation of *K. pneumoniae* for reference purposes. Similarly, a reference series was challenged by intraperitoneal injection of VEE virus.

Pathogenesis

With each exposure system, mice were exposed to aerosols of either *K. pneumoniae* or VEE virus. Ten mice were killed at each of four intervals after exposure; five were evaluated for the presence of microorganisms in selected tissues, and five were analyzed for histopathological alterations during infection. Unexposed and sham exposed mice served as controls. Portions of tissue specimens to be tested for the presence of bacteria or viruses were homogenized to 5% or 10% (w/v) suspensions in the same medium used for aerosol collection. Homogenization was accomplished with a Model SDT 182N Tissuemizer (Tekmar Co., Cincinnati, Ohio). The tissue suspensions and blood samples were used as

TABLE II
Comparison of Respiratory Dose Response to Venezuelan Equine Encephalitis Virus for Swiss-Webster Mice Exposed by Three Procedures

Challenge Type	MDTD[A] (> 1.0 LD_{50} Dose)	LD_{50}[B] (95% CL[C])
Nose-Only	7.1 - 9.6	8 (5-12)
Whole-Body	7.1 - 9.5	11 (8-16)
Intraperitoneal	8.0 - 8.7	2 (1-4)

[A]Mean days to death, geometric mean.
[B]Expressed as plaque forming units (pfu)
[C]Confidence limit.

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inocula for assay. Tissues for histopathologic examination were fixed in a solution of 10% formaldehyde in phosphate-buffered saline (pH 7.0), embedded in paraffin, sectioned at 6 μm and routinely stained with hematoxylin and eosin. Selected tissue sections were stained by special procedures as needed.

Results

Respiratory Dose-Response

For *K. pneumoniae* the median lethal dose (LD_{50}) for nose-only exposure was 335 cfu, which was significantly less than the 3741 cfu required by whole-body exposure (Table I). With intranasal instillation the LD_{50} value was 6 cfu, which corresponds to previous observations.[3,10] Mean time to death correlated with the relative sensitivity to infection by the different exposure systems.

Mice have been shown to be exceptionally sensitive to lethal infections of VEE virus. Consequently, the LD_{50} and mean time to death values obtained for nose-only exposure, whole-body exposure and intraperitoneal injection were quite similar (Table II). If the inhaled dose is corrected to an estimated retained dose of 50%,[11] the LD_{50} values are essentially identical: nose-only, 4 plague forming units (pfu); whole-body, 5 pfu; intraperitoneal injection, 2 pfu. Additionally, the 95% confidence limits for each exposure type were within a limited range.

Diminution of viability and/or infectivity of the biological agent disseminated in aerosol is expressed as the spray factor.[12] The spray factors for replicates of each exposure system-agent combination exhibited minimal variation (Table III). The spray factor for the whole-body exposure system, however, consistently was 0.3 to 0.5 $\log_{10}$ greater than the corresponding loss encountered with the nose-only module. With both exposure systems, the decrement in infectious agents was approximately 1.0 $\log_{10}$ greater for *K. pneumoniae* as contrasted with VEE virus.

TABLE III
Comparison of Spray Factors for *Klebsiella pneumoniae* and Venezuelan Equine Encephalitis Virus Obtained with Nose-Only and Whole-Body Aerosol Exposure Systems

Test Replicate	Spray Factor (− $\log_{10}$) Nose-Only	Whole-Body	Difference[A]
K. pneumoniae			
1	6.03	6.44	0.41
2	6.02	6.54	0.52
3	6.37	6.68	0.31
Mean	6.16	6.58	0.42
VEE Virus			
1	5.13	5.56	0.43
2	4.81	5.24	0.43
3	5.36	5.41	0.05
Mean	5.10	5.40	0.30

[A]Whole-Body Spray Factor versus Nose-Only Spray Factor.

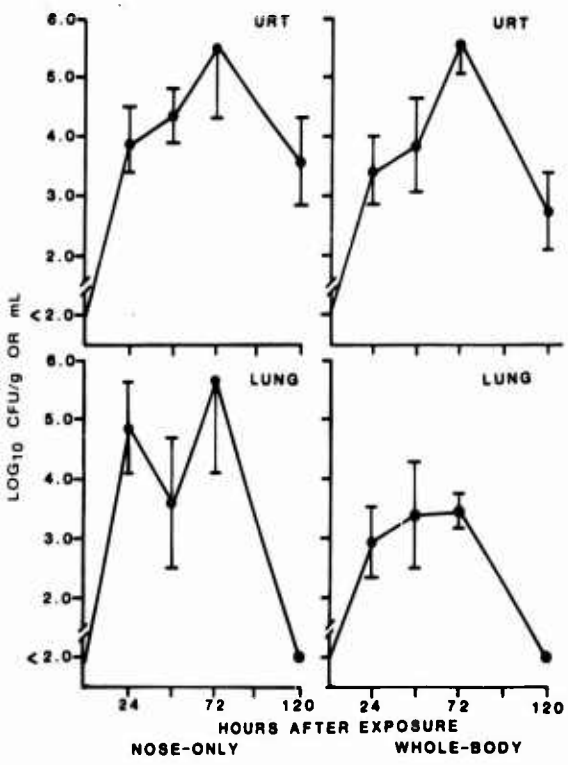

Figure 3 — Concentrations of *Klebsiella pneumoniae* recovered from the upper respiratory tract (URT) and lungs of Swiss-Webster mice after aerosol challenge using a nose-only system and a whole-body system. Each point represents the geometric mean (± SEM) for five mice.

Pathogenesis

Pathogenesis studies were designed to expose the mice in each exposure system, nose-only and whole-body, to equivalent LD_{50} concentrations of infectious agent; the target inhaled dose was 10.0 LD_{50}. The inhaled dose of *K. pneumoniae* received was 1.4 LD_{50} (480 cfu) by nose-only exposure and 0.4 LD_{50} (1640 cfu) by whole-body. This level of challenge induced a limited, instead of fulminating, *Klebsiella* infection. Only 20% of the mice died (nose-only 15/60, whole-body 9/60) with deaths occurring between days 3 and 5. None of the mice used for bacteriology and pathology evaluations were moribund when killed; however, *Klebsiella*-infected mice characteristically exhibit few overt clinical signs until immediately prior to death.

By 24 hr after nose-only exposure, concentrations of *K. pneumoniae* approximating 10^1 bacteria/g of tissue were present in the upper respiratory tract (URT) and lungs (Figure 3). The concentrations continued to increase until 72 hr, when resolution of the infection began. By contrast, the detection of bacteria in the lungs was delayed in mice given whole-body exposure, and the number of organisms in the lungs was drastically less. Bacteremia was an inconsistent observation during the course of infection regardless of the

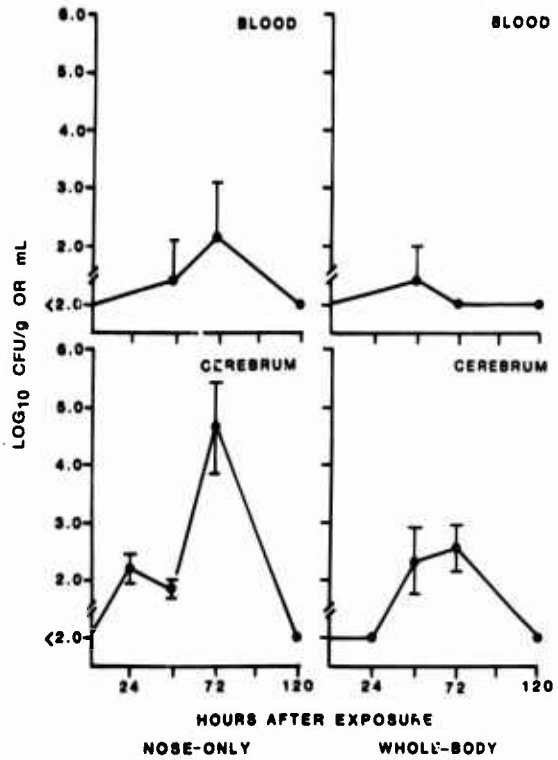

Figure 4 — Concentrations of *Klebsiella pneumoniae* recovered from the blood and cerebrum of Swiss-Webster mice after aerosol challenge using a nose-only system and a whole-body system. Each point represents the geometric mean (± SEM) for five mice.

exposure system (Figure 4). Significant numbers of bacteria ($10^{4.6}$/g) were detected only at 72 hr in the cerebrum of nose-only exposed mice; minimal concentrations of bacteria ($< 10^{2.5}$/g) were present in the cerebrum at any time interval after whole-body exposure. With both exposure systems, bacterial concentrations in the spleen and mesenteric lymph nodes began increasing by 36 hr, reached a zenith at 72 hr and receeded to nondetectable levels by 120 hr (Figure 5). Consistently the levels of bacteria were higher in animals exposed with the nose-only system.

Pathological alterations induced by *K. pneumoniae* were essentially identical for the two aerosol exposure systems. Mild suppurative rhinitis was the most important lesion observed in infected mice. A mild suppurative bronchopneumonia, accompanied by large accumulations of bacteria in the alveoli, was evidenced in an occasional lung lobe of all animals by 48 hr after exposure but was resolving by 72 hr.

Infection with VEE virus was initiated with inhaled doses of 18.0 to 22.0 LD_{50} of virus (179-198 pfu). All mice killed for examination at 48 hr were clinically ill; mice at the other time intervals were moribund. Deaths occurred between days 6 and 8 after exposure.

Virus concentrations in the blood and each of the tissues (URT, lungs, cerebrum, spleen and mesenteric lymph nodes) reached maximum titers 24 to 36 hr earlier for mice exposed by nose-only than for mice exposed to whole-body aerosols (Figures 6, 7, 8). Concentrations of virus in the URT and lungs approached 10^8 pfu/g of tissue. Viremia was greatest, 10^7 pfu/g, 48 to 96 hr after exposure, the midpoint of the infection. Highest virus concentrations in the cerebrum ($10^{9.5}$ pfu/g) occurred just prior to death. Virus titers in the spleen and mesenteric lymph nodes rose rapidly to $10^{7.5}$ to $10^{8.5}$ pfu/g, then diminished from 96 hr until death.

Lymphoid necrosis involving the white pulp of the spleen and the paracortex of the mesenteric lymph nodes was seen at 48 hr in mice exposed by nose-only as contrasted to 96 hr with whole-body exposure. By 144 hr after exposure, the lymphoid necrosis had subsided and a proliferation of macrophages and lymphoblastic lymphocytes was observed within the necrotic areas. Necrosis of the pancreatic acini was evident in both groups of mice at 96 hr and continued to increase in severity through 144 hr. A nonsuppurative meningoencephalitis, which became evident at 144 hr, increased in intensity until death occurred. The primary areas of central nervous system involvement were the cerebral cortex, hippocampus, thalamus and brain stem. Necrosis of the lymphoid tissues was the probable cause of death. Disease induced by both aerosol systems was identical.

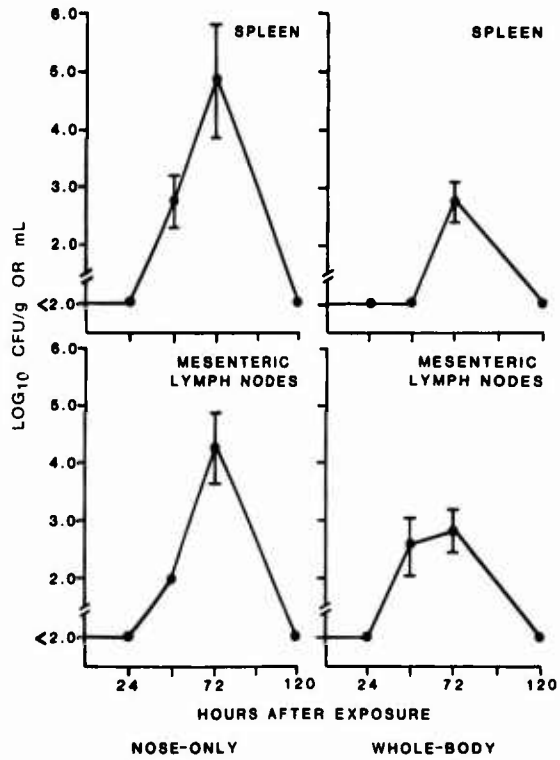

Figure 5 — Concentrations of *Klebsiella pneumoniae* recovered from the spleen and mesenteric lymph nodes of Swiss-Webster mice after aerosol challenge using a nose-only system and a whole-body system. Each point represents the geometric mean (± SEM) for five mice.

Discussion

Differences between the two aerosol delivery systems were more obvious with studies of *K. pneumoniae*, the model that required greater numbers of organisms to induce a lethal response. The increase of 1 $\log_{10}$ in the LD_{50} value for exposure to whole-body aerosol — as contrasted to the LD_{50} by the nose-only mode — was unexpected. Further, mice exposed to whole-body aerosols exhibited a consistent delay in the development of detectable levels of bacteria in the blood and tissues as well as decreased concentrations of bacteria. These observed differences between the two aerosol systems apparently were not associated with an increased diminution in organism viability during aerosolization in the whole-body system, as the spray factors for each aerosol system were similar; albeit a slightly greater loss always was present with the whole-body system. The same relative degree of loss of organism viability occurred with bacterium and virus without an equivalent effect on lethality determinations. Possibly, the deviation is related only to variations in respiratory rate. The authors consistently noted that mice exposed by the nose-only mode exhibited markedly increased respiratory rates when compared with mice exposed in the whole-body unit. The authors did not take such differences into account when they made estimates of inhaled dose using Guyton's formula.[8] Excitation with concomitant faster respiration could result in greater numbers of infectious bacteria being deposited and retained in the bronchioles and alveoli. Another possibility is that during whole-body exposure a greater surface area other than the respiratory tract is available on which the bacteria can be deposited. With whole-body exposure, bacteria can be deposited on the animal's body, the cage and any other available surfaces, thereby decreasing the delivered dose to the respiratory tract.

In the two aerosol systems, factor(s) that caused the differences in lethality and pathogenesis of *K. pneumoniae* did not impact on evaluations of the highly virulent VEE virus model. Yet an elevated rate of respiration may have been associated with maximum virus titers being reached 24 to 36 hr earlier for mice exposed by the nose-only method. Why the large difference observed between the two aerosol systems with *K. pneumoniae* was undetected with VEE virus is strictly speculative, although it was a common feature. The disparity between organisms may be a direct correlate of relative virulence; the greater the degree of lethality, the less the effect that can be exerted by different exposure systems. One also must consider the effect of the suspending medium used to generate the aerosols as related to the potential of enhancing impingement of microorganisms onto various surfaces. Trypticase soy broth, as used for *K. pneumoniae*,

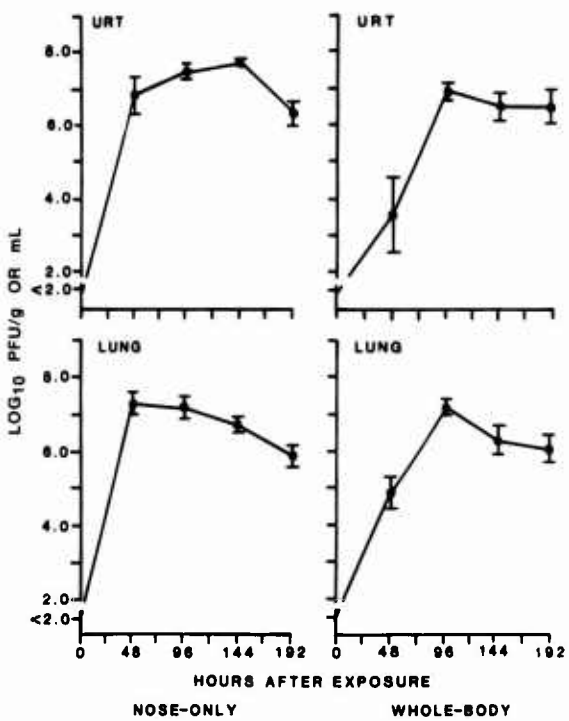

Figure 6 — Concentrations of Venezuelan equine encephalitis virus recovered from the upper respiratory tract (URT) and lungs of Swiss-Webster mice after aerosol challenge using a nose-only system and a whole-body system. Each point represents the geometric mean (± SEM) for five mice.

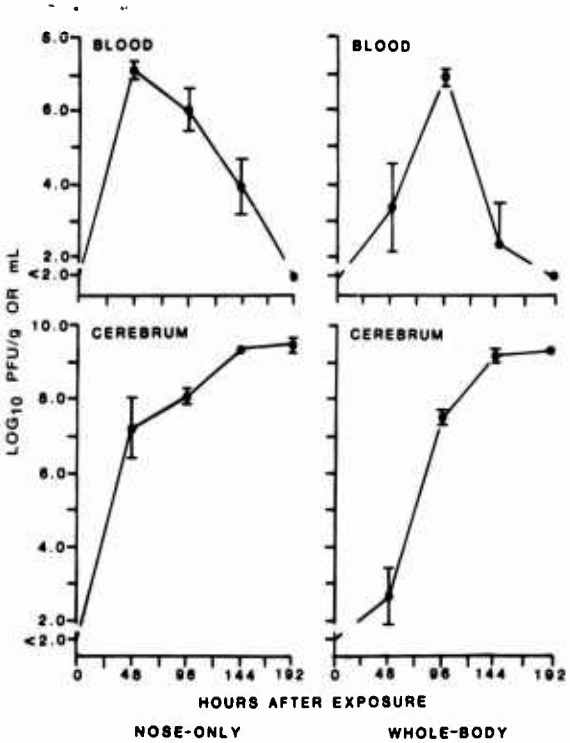

Figure 7 — Concentrations of Venezuelan equine encephalitis virus recovered from the blood and cerebrum of Swiss-Webster mice after aerosol challenge using a nose-only system and a whole-body system. Each point represents the geometric mean (± SEM) for five mice.

may induce increased bacterial adherence to surfaces. By contrast, EMEM supplemented with FBS, the diluent for VEE virus, may exert little or no added impingement effects.

In contrast to the bacterial distribution and the pathological alterations evidenced with a fulminating *K. pneumoniae* infection in mice,[3] minimal numbers of bacteria were detected in tissues other than the respiratory tract, and the most important pathological lesion noted was mild suppurative rhinitis. Bronchopneumonia developed only sporadically. The limited infection was resolving by 72 hr after exposure and essentially was eliminated within 120 hr. Thus, in the absence of an overwhelming challenge, the mice were able to suppress *K. pneumoniae* respiratory infections via innate defense mechanisms prior to the presence of detectable antibodies. The mouse, therefore, would be a better animal model than the rat[2] in which to study *K. pneumoniae* pathogenesis. Disease induced in Swiss-Webster mice by VEE viruses in small-particle aerosol was identical to that observed in mice and hamsters infected by parenteral injection[13,20] and hamsters exposed to whole-body aerosol (E. H. Stephenson, Unpublished Data).[21] The disease is acute and bimodal in nature, involving primarily the reticuloendothelial system but also affecting the central nervous system in the terminal stages. Infectious virus traversed the respiratory mucosa, induced a viremia, then commenced replication in the lymphoid tissues. Replication in the central nervous system, with development of meningoencephalitis, occurred during the latter phase of the disease. In the naive animal, there was no indication that VEE virus infected the brain via the olfactory route as previously proposed.[22]

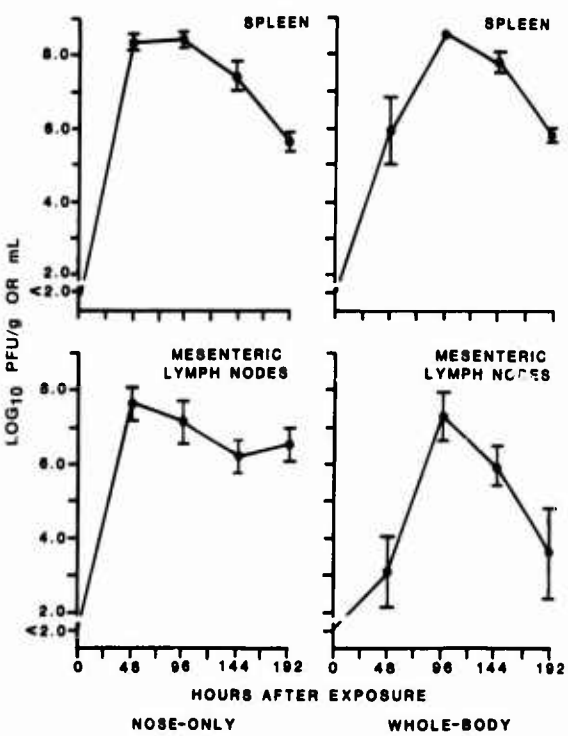

Figure 8 — Concentrations of Venezuelan equine encephalitis virus recovered from the spleen and mesenteric lymph nodes of Swiss-Webster mice after aerosol challenge using a nose-only system and a whole-body system. Each point represents the geometric mean (± SEM) for five mice.

Availability of both nose-only and whole-body aerosol exposure systems provides added dimensions to the investigations of airborne transmitted diseases. Each system is superior to the other in specific areas. For nose-only exposure the advantages include the following: 1) aerosol exposure is limited to the respiratory tract, thereby reducing infections caused by alternate routes of entry; 2) the entire body of the animal is not exposed to the aerosol, thereby precluding secondary exposure via preening; and 3) a higher challenge dose is obtained with less substrate. The whole body system, on the other hand, exhibits the following characteristics: 1) the exposure better simulates aerosol challenges as they occur in the laboratory or environment; 2) the use is not limited to obligate nasal breathing animals; 3) the loading of animals into the unit is easier and less complicated; and 4) the system accommodates varying sizes and mixed species of animals for simultaneous exposure. Depending on the objectives of a given study, an aerosol exposure system can be selected that will best meet the criteria imposed and provide the desired effect.

Acknowledgments

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The 1987 supplement to the *NIOSH Manual of Analytical Methods, 3rd ed.* is now in press and will be mailed to subscribers in March or April 1988 by the U.S. Government Printing Office (telephone 202/783-3238). Fifty-one revised methods are included, in loose-leaf format. Important changes included in this supplement include changes to the analytical methods for isocyanates and for ethylene oxide.

Of particular importance is the withdrawal of Method 5505 for the Isocyanate Group and the recommendation to use Method 2535 for toluene-2,4-diisocyanate. The issues surrounding this recommended change can be found in Teass, A.W., and M.J. Seymour, *Applied Industrial Hygiene*, vol. 2, p. 182 (1987).

The recommended method for ethylene oxide has been changed from Method 1607 to Method 1614 (with 3702 as a field-readable alternative) because it is more stable. These changes roughly correspond to changing from OSHA Method #30 to OSHA Method #50.

This supplement to the manual also includes a reference to "Recommendation for Prevention of HIV Transmission in Health-Care Settings," *Morbidity & Mortality Weekly Report*, vol. 36, p. 25, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control (August 21, 1987).

The purpose of this citation is to alert laboratories to procedures published by the Centers for Disease Control that should be observed when analyzing blood and tissue samples.